Original Article

Expression of NC-2 Receptor on MCL Cells and Its Natural Cytotoxicity Against Cancer Cells

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ABSTRACT

Objectives: To identify the expression of NC-2 on an interleukin-3 dependent mast cell line (MCL) and investigate the activity of this receptor against tumor cells
Design: Laboratory study
Setting: Cellular and Molecular Center, Shahrekord, Iran
Subjects and Methods: The MCL cells were stained with D9 monoclonal antibody (anti-NC-2) and analysed by flow cytometry. This was confirmed by immunoperoxidase staining. The cytotoxicity assay was performed to show the cytotoxic activity of MCL cells against 51Cr-labelled WEHI-164 tumor cells.
Intervention: The expression of NC-2 on MCL cells, and the anti-tumor activity of this receptor were investigated.
Main Outcome Measure(s): Flow cytometric analysis and *in vitro* experiments were performed for showing the activity of NC-2 against cancer.
Results: NC-2 receptor was expressed on more than 95% of MCL cells. Pretreatment with D9 monoclonal antibody resulted in about 63% reduction in natural cytotoxicity of MCL cells against WEHI-164 tumor target cells.
Conclusions: NC-2 is also one of the receptors expressed on MCL and utilized for WEHI-164 tumor cell killing.

KEY WORDS: monoclonal antibody, natural cytotoxicity, receptor, tumor

INTRODUCTION

Natural cell-mediated cytotoxicity (NCMC) is a major component of innate cellular immunity against cancer and infection. NCMC is mediated primarily by leucocytes which perform natural killing (NK) and natural cytotoxicity (NC) without the requirement for prior sensitization and there is no immunological memory associated with this type of responses. This mechanism is characteristically and functionally different from the cell-mediated cytotoxicity of cytotoxic T lymphocytes. Cells mediating NK and NC are two distinct and probably related NCMC effector mechanisms which are distinguishable from each other by the time differences required to mediate their killing, as well as the kinetics of their appearance and decline in mice[1-3].

Natural killer (NK) cells belong to an important lymphocyte population that eliminates transformed cells and invading viral pathogens without any prior sensitization. These cells possess not only natural killing activity against non-self and altered-self cells but also exhibit cytokine production and antibody-dependent cell-mediated cytotoxicity (ADCC)[4]. It has been shown that NK cells might not serve merely as cytotoxic lymphocytes combating viral pathogens and malignant tumors, but must also be considered as important immunoregulatory cells with a significant influence on adaptive immunity[5]. The effector's functions of NK cells are regulated by integrated signals across the array of stimulatory and inhibitory receptors engaged upon interaction with target cell surface ligands[6]. Intensive research during the 1990s has defined a large number of activating and inhibitory receptors[7-9]. NK cells are heterogeneous in their receptor repertoire, in the sense that different cells express different combinations of activating and inhibitory receptors. In addition, a functional heterogeneity is emerging, at least in the human. The majority of blood NK cells express moderate levels of CD56 in combination with various molecules of killer cell immunoglobulin receptor (KIR) family[10]. In addition, there is a small subpopulation of blood NK cells that express high levels of CD56 in combination with the inhibitory receptor NKG2A, and no receptors of the KIR family. This subset has low perforin levels and seems to be specialized for high cytokine secretion rather than

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direct killing. The latter subset may be the only NK cells present in the lymph nodes, an organ that was initially thought to be completely devoid of NK cells[11]. Human NK cells can be divided into two functional subsets based on their surface expression of CD56; CD56 (bright) immunoregulatory cells and CD56 (dim) cytotoxic cells. The importance of early hematopoietic growth factors, such as c-kit ligand and flt-3 ligand, and their synergy with IL-15 in the development of human NK cells in the bone marrow has permitted the investigation of novel cytokine combinations for optimizing *in vivo* expansion of NK cell in the clinic. The importance of lymph nodes as a site for NK cell development has recently been elucidated[12].

The major differences between NK and NC are the receptors and mechanisms involved in cytotoxicity[13-15]. The initial step in the mechanism of cytotoxicity of NK and NC is the recognition and binding of effector cells to tumor cells via receptor-ligand interactions. A number of these receptors have been identified on human and rodent leucocytes mediating NK cytotoxicity. To date, however, only two such receptors, NC-1.1 and NC-2 were recognized[16,17]. NC-1.1 was identified by a mouse anti-mouse monoclonal antibody (mAb) 1C4 (anti-NC-1.1). It is a monomeric phosphoprotein of 45,000 MW expressed on the surface of predominantly large and granular leucocytes of different hemopoietic cell lineages[16]. Flow cytometric analysis showed that NC-1.1 is expressed on less than 5% of fresh CBA mouse spleen cells and 20-50% of CBA- interleukin-3 cells. *In vitro* treatment of spleen cells from a number of inbred mouse strains with anti-NC-1.1 (1C4), markedly decreased NC activity of spleen cells against 51Cr-labeled WEHI-164 targets[16]. Administration of a single dose of 1C4 in a number of mouse strains depletes NC activity from the spleens of mice for at least one week, with the maximal effect occurring 24 hours after treatment[18]. NC-2 was identified by a rat anti-mouse monoclonal antibody D9 (anti-NC-2). It is a 50,000 MW molecule expressed on the surface of mainly large and granular leucocytes in mice. This receptor is expressed on < 6% of splenic leukocytes of different inbred mouse strains. Pretreatment of (CBA × C57BL/6) F1 mouse spleen cells with different doses of mAb D9 *in vitro* blocked NC against WEHI-164, whereas NK activity against YAC-1 was not affected[17]. By culturing CBA mouse spleen cells in interleukin-3 conditioned media, a stable cell line called mast cell line (MCL) was generated[19]. It has been shown that this cell line has high natural cytotoxicity against WEHI-164 tumor target cells and was used as a NC like cell line to characterize natural cytotoxic cells. These cells expressed NC-1.1 receptor but not characteristic cell surface markers of T, B lymphocytes, macrophages, or NK cell. Pretreatment of MCL cells with anti-NC1.1 antibody blocked the NC activity of these cells by approximately 70%[19,20] .

This article reports the expression of NC-2 on MCL cells, and the investigation of the activity of this receptor.

MATERIAL AND METHODS

Tissue culture medium (TCM)

The study was conducted in the cellular and molecular research center at Sharekord University of Medical Sciences, Iran. Approval of the ethical committee was obtained. Dulbecco's modification of eagles medium (CSL, Melbourne, Victoria, Australia) was supplemented with 20 mm HEPES, 2 mM L- glutamine, 50 μM 2-mercaptoethanol, 0.15% sodium bicarbonate, 50 μg/ml gentamicin and 10% fetal calf serum (FCS). The supplemented TCM was then further conditioned either with IL-3 for growth of MCL or with IL-2 for C57BL/6 IL-2 dependent cell lines. Cell lines and monoclonal antibodies.

NK-like MCL and NK-like C57BL/6-interleukin-2 (IL-2) dependent cell lines were generated[19,20]. WEHI-164 (a BALB/c fibrosarcoma) was provided by Walter and Elisa Hall Institute, Melbourne, Australia. The mAbs used were anti-NC-2 mAb D9 (rat IgG2a) and anti–CD32/CD16 (rat IgG2b; clone 2.4G2, anti-Fc gamma receptor RII/RIII).

Flow cytometric analysis

MCL (NC like) and C57BL/6-IL2 (NK like) cells were harvested and washed in PBS containing 1% FCS at $5x10^5$ cells/ml. One hundred micro liter of each cell suspension were aliquoted into each staining tubes. After blocking Fc receptors with 2.4G2 mAb, the cells were incubated either with 100 μl biotinylated D9 mAb and rat IgG2a isotype control mAb for 30 min on ice, followed by a further incubation with streptavidin–fluoroscein isothiocyanate (FITC) complex (Amersham, Buckinghamshire, UK). After two washes, the cells were re-suspended in 250μl PBS and analyzed in the FACScan automated flow cytometer using the CELL QUEST software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Five thousand cells were acquired for analysis in flow cytometer[20].

Immunoperoxidase Staining techniques

Fresh MCL cells were cytocentrifuged onto gelatin coated slides. The specimens were air dried and fixed in acetone for 10 minutes. The fixed cells were incubated with 100 μl of pretitred anti-Fc gamma receptor (2.4G2) for 30 minutes.

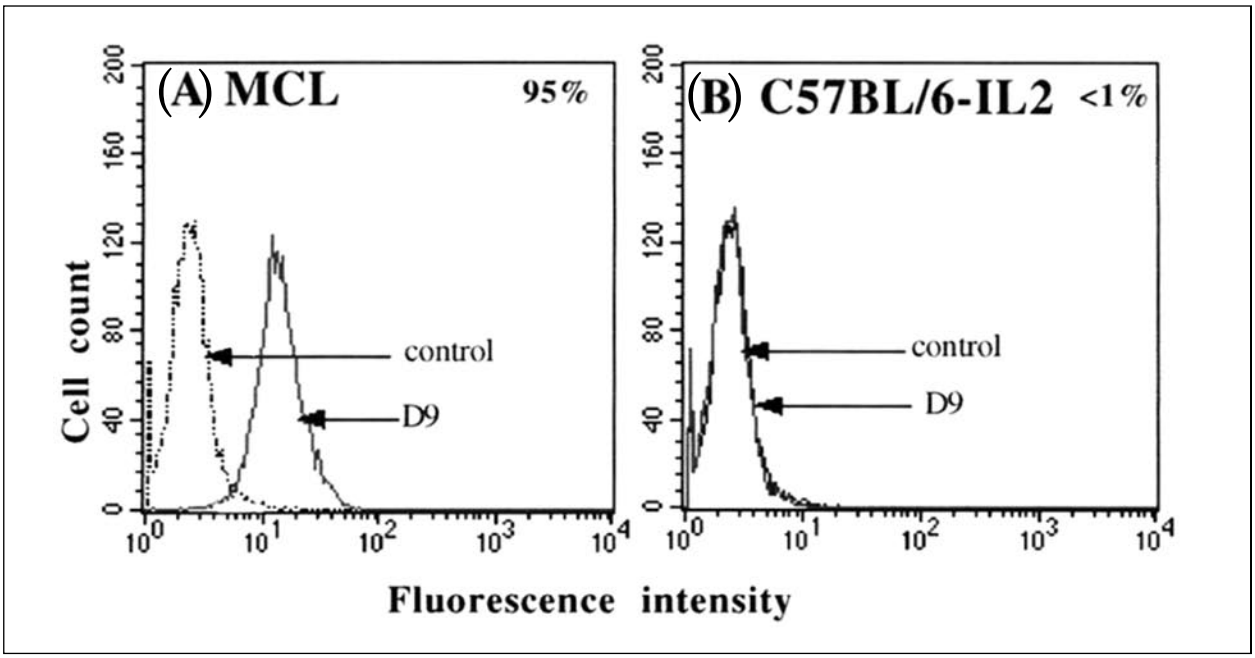

Fig. 1: Flow cytometric analysis showing expression of NC-2 receptor on (A) MCL (NC like) but not on (B) C57BL/6-IL2 (NK like) cell line. The cells were first incubated with mAb 2.4G2 (anti-CD32/CD16) and then with mAb D9 (anti-NC-2). (...) fluorescence histograms of cells stained with rat IgG2a isotype mAb and (—) cells stained with mAb D9.

The slides were washed with PBS pH 9.6 and incubated with 100 μl of pretitered mAb D9 in a humid box at room temperature for 30 minutes. Endogenous peroxidase activity was quenched in a 2% w/v hydrogen peroxidase/methanol bath for 10 minutes. The specimen were then incubated with 100 μl strepavidin-conjugated Horse-Radish-Peroxidase conjugate (Amersham, Buckinghamshire, UK), followed by reaction with 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St Louis, MO,USA) for not more than 10 minutes. The cells were washed with tap water and counter stained with Carazzi,s hematoxylin (Histo-Lab Fronine, NSW, Australia) with replacement of slides into Carazzi,s hematoxylin for two minutes, followed by rinsing in tap water. The slides were then dipped in 1% acid alcohol for five seconds and then transferred into Scott's tap water for two minutes. The slides were then dehydrated through three lots of 70% and two of 100% ethanol and then mounted with natural mounting medium (Ajax Chemicals, Australia) for microscopic analysis.

Cytotoxicity assay

NC activity was assayed by *in vitro* lysis of ^{51}Cr-labelled WEHI-164, as previously described[19]. The experiment was conducted in quadruplicate in 200 μl TCM in 96-well micro-titer trays. Briefly, MCL cells were incubated with ^{51}Cr-labelled targets at effector / target (E : T) ratios in the range of 100:1-12.5:1. The cells were incubated at 37 ^{o}C / 5% CO_2 for 18 hr. Supernatants (100 μl) from each well were harvested and the level of radioactivity was measured in a COBRA gamma-counter (Packard Instrument CO., Downer's Grove, Illinois). The results were first calculated as percentage specific lysis:

$$\text{Percent specific lysis} = \frac{\text{CPM (Sample) - CPM (Background)} \times 100}{\text{CPM (Total Release) - CPM (Background)}}$$

Background count per million (CPM) was obtained from wells containing target cells alone and total release was determined by counting 51Cr-labelled target cells. Lytic units (LU) were calculated from the linear portion of a graph of percentage specific lysis versus E:T ratios. One LU is herein defined as the number of effector cells which mediate 20% specific lysis of the target cells. Results are expressed either as LU per 10^8 MCL or percentage reductions in LU according to the following formula:

$$\text{Percent reduction in LU} = \frac{\text{LU (control)- LU (D9 treated)} \times 100}{\text{LU (control)}}$$

For antibody blocking studies, the MCL cells were pretreated with mAb D9 or isotype matched mAb, washed and resuspended to the original volume prior to use in the cytotoxicity assay[21].

RESULTS

To show the expression of NC-2 on MCL and NK-like C57BL/6-IL2 cells, flow cytometric analysis was performed. Results showed that mAb D9 (anti NC-2 receptor) bound to more than

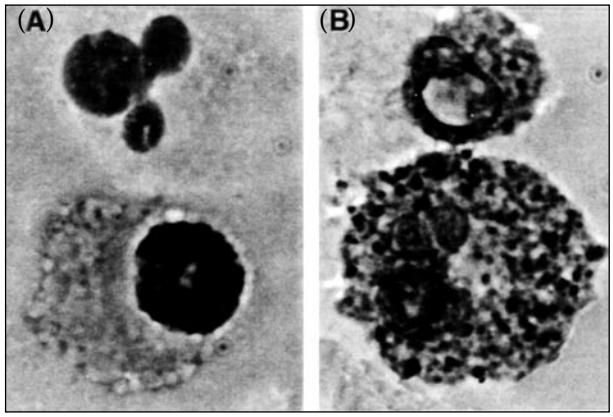

Fig. 2: Immunoperoxidase staining of MCL cells: Cytocentrifuged MCL cells were stained with either IgG2a Isotype matched mAb (A) or mAb D9 (B).

95% of NC-like MCL cells, but not to the NK- like C57BL/6-IL2 NK-like cells (Fig. 1). To confirm the flowcytomery finding, immunoperoxidase staining technique was carried out on fresh MCL cells; results approved the flow cytometric finding (Fig. 2).

Natural cytotoxic activity of MCL cells was assayed by *in vitro* lysis of ^{51}Cr-labelled WEHI-164 cells (NC tumor target). Results demonstrated the maximal lysis of WEHI-164 target at 18 hours in the cytotoxicity assay. The lytic units of MCL cells were reduced by approximately 63% following pretreatment of cells with mAb D9 compared to the control cells treated with IgG2a isotype matched mAb (Fig. 3).

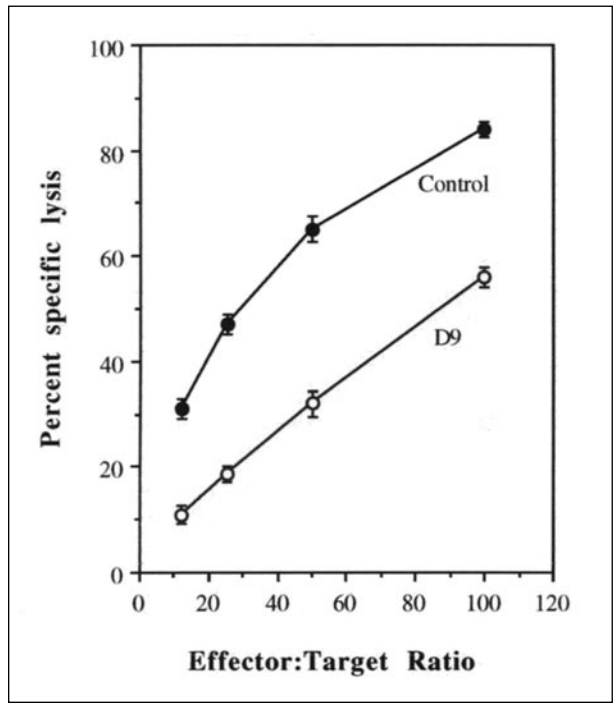

Fig. 3: Effect of *in vitro* treatment of mAb D9 on the NC activity of MCL cells: Cytotoxic activity of MCL cells was assayed by *in vitro* lysis of ^{51}Cr-labelled WEHI-164 cells. The lytic units of MCL cells were reduced by approximately 63% following pretreatment of cells with mAb D9 compared to the control cells treated with IgG2a isotype matched mAb.

DISCUSSION

Our flow cytometry results showed that NC-2 receptor is expressesd on about 95% of MCL cells, this was confirmed by immunoperoxidase staining technique. Furthemore, in a ^{51}Cr-release assay, anti-NC-2 antibody (D9) blocked 63% of NC activity *in vitro*. Previous study showed that NC-2 is expressed on about 6% of (C57BL/6 × CBA) F1 and BALB/c and about 4% of C57BL/6 and CBA mice spleen cells. Anti-NC2 antibody blocked 60% of NC activity of splenic leukocytes in a cytotoxicity assay [17]. It has been previously reported that NC-1.1 is a molecule expressed on spleen cells of different mouse strains[14,16] and as reported in our previous publication the NC-1.1 receptor is also presents on MCL cell line[19]. Further studies showed additional effects of anti-NC-2 (D9) and anti-NC-1.1 (1C4) on cytotoxicity of mice spleen cells and suggested that NC-1.1 and NC-2 are two different receptors on mouse spleen cells. Western blot analysis of affinity purified NC-1.1 and NC-2 indicated that the receptor identified by mAb D9 (anti-NC-2) is not the previously described NC-1.1[17]. Co-expression of NC-1.1 and NC-2 on granular splenic leukocytes of (C57BL/6 X CBA) F1 mice was examined by dual color flow cytometry. Results showed that all NC-2$^+$ granular leukocytes co-expressed NC-1.1 whereas the converse was not true[17]. Our present finding suggests that, similar to previous reports on different inbred mouse strains, NC-1.1 and NC-2 are two distinct receptors which are expressed on MCL cells. Furthermore, it is possible that in MCL cells population there is a group of cells which may have both the receptors, which in turn probably abrogates the cytotoxicity of each individual receptor[22].

Recent studies on NK cells have identified multiple receptors which recognize ligands on the surface of target cells. These receptors belong to two multi-gene families, NKR-P1 and Ly-46, which display either activation or inhibition effects on natural killing[13]. Upon ligation to its specific ligand the receptor either transduces a positive signal to the effectors to kill the target cell or a negative signal to turn off the killing thus providing a delicate regulatory mechanism to control cytotoxicity[14].

Phosphorylation studies of NC-1.1 showed that key intracellular signaling pathway involving protein kinase C (PKC) and protein kinase G (PKG) interact to effect a coordinated control of NC. The fact that increase in phosphorylation up-regulates NC argues for NC-1.1 to be an activated receptor[14]. Biochemical studies to determine whether NC-2 is

also a signaling receptor are in progress, and data to date suggest that NC-2, like NC-1.1, may also be an activation receptor. In order to achieve a balanced control in the regulation of NC, it is likely that inhibitory receptors also exist on leukocytes mediating natural cytotoxicity.

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REFERENCES

1. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene 2008; 27:5932-5943.
2. Kiessling R, Haller O. Natural killer cells in the mouse:an alternative immune surveillance mechanism?. Contemp Top Immunobiol 1978; 8:171-201.
3. Stutman O, Paige CJ, Figarella EF. Natural cytotoxic cells against solid tumors in mice. I. Strain and age distribution and target cell susceptibility. J Immunol 1978; 121:1819-1826.
4. Iizuka S, Kaifu T, Nakamura A, Obinata M, Takai T. Establishment and functional characterization of novel natural killer cell lines derived from a temperature-sensitive SV40 large T antigen transgenic mouse. J Biochem 2006; 140:255-265.
5. Woo CY, Clay TM, Lyerly HK, Morse MA, Osada T. Role of natural killer cell function in dendritic cell-based vaccines. Expert Rev Vaccines 2006; 5:55-65.
6. Zhang C, Zhang J, Wei H, Tian Z. Imbalance of NKG2D and its inhibitory counterparts: how does tumor escape from innate immunity? Int Immunopharmacol 2005; 5:1099-1111.
7. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol 2001; 22:633-640.
8. Ferlazzo G, Thomas D, Lin SL, *et al*. The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. J Immunol 2004; 172:1455-1462.
9. Moretta A, Vitale M, Bottino C, *et al*. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti -p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. J Exp Med 1993; 178:597-604.
10. Moretta A, Bottino C, Vitale M, *et al*. Activating receptors and co receptors involved in human natural killer cell – mediated cytolysis. Annu Rev Immunol 2001; 19:197-233.
11. Yokoyama WM, Plougastel BF. Immune functions encoded by natural killer gene complex. Nat Rev Immunol 2003; 3:304-316.
12. Farag SS, Caligiuri MA. Human natural killer cell development and biology. Blood Rev 2006; 20:123-137.
13. Yokoyama WM. Recognition structures on natural killer cells. Curr Opin Immunol 1993; 5:67-73.
14. Holmgreen SP, Wang X, Clarke GR, *et al*. Phosphorylation of the NC-1.1 receptor and regulation of natural cytotoxicity by protein kinase C and cyclic GMP-dependent protein kinase. J Immunol 1997; 158:2035-2041.
15. Porgador A. Natural cytotoxicity receptors:pattern recognition and involvement of carbohydrates. Scientific World Journal 2005; 5:151-154.
16. Smart YC, Stevenson KL, Farrelly ML, Brien JH, Burton RC. Production of a monoclonal allo-antibody to murine natural cytotoxic cells. Immunol Cell Biol 1990; 68:277-284.
17. Shirzadeh H, Burton RC, Brien JH, Smart YC. Monoclonal antibody anti-NC-2 identifies a second receptor on cells mediating natural cytotoxicity in mice. Immunology 1998; 93:122-128.
18. Smart YC, Farrelly ML, Burton RC. Correlation of growth of tumors in NC-cell-depleted mice with NC- and NK-cell-mediated lysis in vitro. Int J Cancer 1992; 50:817- 821.
19. Shirzadeh H, Clarke GR, McNeil HP, Wang H, Burton RC, Smart YC. An IL-3 induced splenic NC-1.1+ mast cell line mediates natural cytotoxicity independent of TNF-alpha. Cell Immunol 1996; 174:147-154.
20. Brien JH, Smart YC, Farrelly ML, Burton RC. Phenotype and morphology of murine NC-1.1+ natural cytotoxic cells. Immunol Cell Biol 1994; 72:161-168.